CHEMISTRY INVESTIGATORY PROJECT
ON

CHROMATOGRAPHY AND ITS MECHANISM

Submitted By
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Understanding Mechanisms Underlying the Process of Chromatography and Hence Studying Out its Applications

Thesis submitted for practical evaluation in AISSCE 2018-2019 in chemistry

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Roll: ……………..
Kendriya Vidyalaya Mankhurd
2018-2019

तत् त्वं पूषन् अपावृणु
केन्द्रीय विद्यालय संगठन

DEPARTMENT OF CHEMISTRY
KENDRIYA VIDYALAYA MANKHURD
NAUSENA BAUG, MUMBAI – 400094

CERTIFICATE

This is to certify that SRIJON SADHUKHAN, a student of class XII B, has successfully completed the research on the below mentioned project under the guidance of Mr. RAMENDRA ACHARYA (subject teacher) during the year 2018-2019 in partial fulfillment of chemistry practical examination conducted by AISSCE, New Delhi.

Signature of the external examiner   Signature of the chemistry teacher

ACKNOWLEDGEMENT

I wish to express my deep gratitude and sincere thanks to the Principal, Mrs. Indra Devi Jain of K.V. Mankhurd, for her encouragement and for all the facilities that she provided for this project work. I sincerely appreciate her magnanimity by taking me into her fold for which I shall remain indebted to.

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I can’t forget to offer my sincere thanks to parents and also to my classmates who helped me to carry out this project work successfully and for their valuable advice and support, which I received from them time to time.

Srijon Sadhukhan

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“It is surely no accident that the major advances in the field have coincided with the appearance of new experimental techniques such as paper and ion-exchange chromatography, paper electrophoresis, and countercurrent distribution, peculiarly appropriate to the compounds of this group.”

Lord Alexander R. Todd

“Chromatography is a physical method, a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate.”

1. INTRODUCTION

1.1 Chromatography: A Physical Method of Separation

Chromatography is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the *mobile phase,* which carries it through a structure holding another material called the *stationary phase*. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.

Chromatography is derived from Greek χρῶμα *chroma,* which means "color", and γράφειν *graphein,* which means "to write". Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification. Analytical chromatography is done normally with smaller amounts of material and is for establishing the presence or measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

A brief history of Chromatography

Chromatography was first employed in Russia by the Italian-born scientist Mikhail Tsvet in 1900. He continued to work with chromatography in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll, carotenes, and xanthophylls. Since these components have different colors (green, orange and yellow, respectively) they *gave the technique its name. New types of chromatography developed during the 1930s and 1940s made the technique useful for many separation processes.*

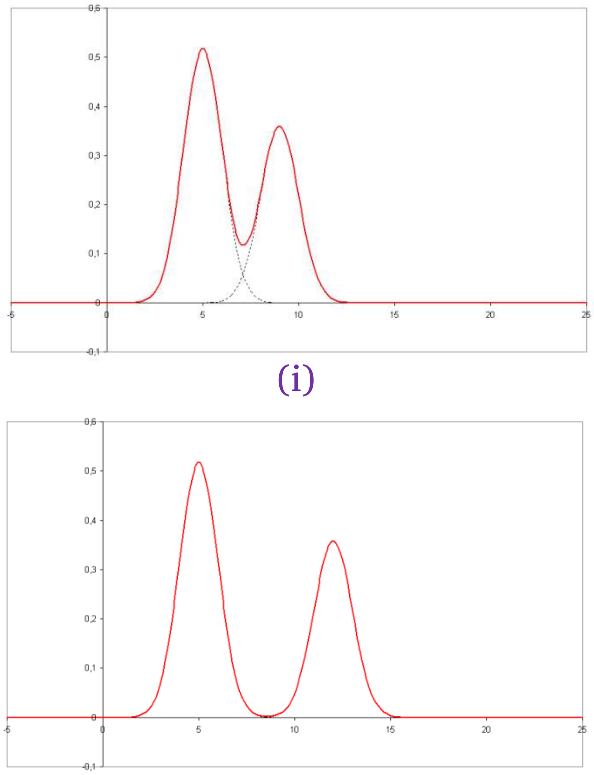
Chromatography technique developed substantially as a result of the work of Archer John Porter Martin and Richard Laurence Millington Synge during the 1940s and 1950s, for which they won the 1952 Nobel Prize in Chemistry. They established the principles and basic techniques of partition chromatography, and their work encouraged the rapid development of several chromatographic methods: paper chromatography, gas chromatography, and what would become known as high-performance liquid chromatography. Since then, the technology has advanced rapidly. Researchers found that the main principles of Tsvet's chromatography could be applied in many different ways, resulting in the different varieties of chromatography described below. Advances are continually improving the technical performance of chromatography, allowing the separation of increasingly similar molecules. Chromatography has also been employed as a method to test the potency of cannabis.

1.2 Chromatography terms

The **analyte** is the substance to be separated during chromatography. It is also normally what is needed from the mixture. **Analytical chromatography** is used to determine the existence and possibly also the concentration of analyte(s) in a sample. A **bonded phase** is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing. A **chromatogram** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.

A **chromatograph** is equipment that enables a sophisticated separation, e.g. gas chromatographic or liquid chromatographic separation. The **eluate** is the mobile phase leaving the column. This is also called effluent. The **eluent** is the solvent that carries the analyte. The **eluite** is the analyte, the eluted solute. An **eluotropic series** is a list of solvents ranked according to their eluting power.

An **immobilized phase** is a stationary phase that is immobilized on the support particles, or on the inner wall of the column tubing. The **mobile phase** is the phase that moves in a definite direction. It may be a *liquid* (LC and Capillary Electrochromatography (CEC)), a *gas* (GC), or a supercritical fluid (supercritical fluid chromatography, SFC).



(i)

(ii)

Figure 1.1 Plotted on the x-axis is the retention time and plotted on the y-axis a signal (for example obtained by a spectrophotometer, mass spectrometer or a variety of other detectors) corresponding to the response created by the analytes exiting the system. In the case of an optimal system the signal is proportional to the concentration of the specific analyte separated. Graphs are on chromatogram with (i) unresolved peaks (ii) two resolved peaks.

The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In the case of HPLC the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or a polar solvent such as methanol in reverse phase chromatography and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.

Preparative chromatography is used to purify sufficient quantities of a substance for further use, rather than analysis. The **retention time** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.

The **sample** is the matter analyzed in chromatography. It may consist of a single **component** or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the **sample** whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as **waste**.

The **solute** refers to the sample components in partition chromatography. The **solvent** refers to any substance capable of solubilizing another substance, and especially the liquid mobile phase in liquid chromatography.

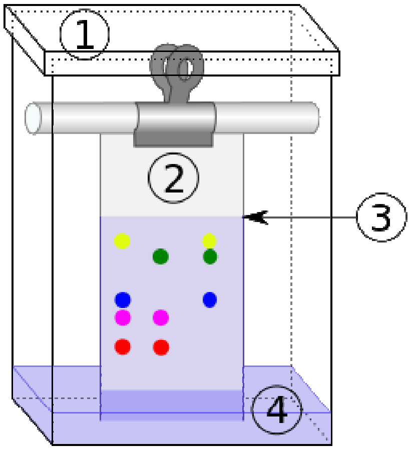
The **stationary phase** is the substance fixed in place for the chromatography procedure. Examples include the silica layer in thin layer chromatography. The **detector** refers to the instrument used for qualitative and quantitative detection of analytes after separation.

Chromatography is based on the concept of partition coefficient. Any solute partitions between two immiscible solvents. When we make one solvent immobile (by adsorption on a solid support matrix) and another mobile it results in most common applications of chromatography. If the matrix support, or stationary phase, is polar (e.g. paper, silica etc.) it is forward phase chromatography, and if it is non-polar (C-18) it is reverse phase.

Planar chromatography is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The **specific retention factor** (R_f) of each chemical can be used to aid in the identification of an unknown substance.

1.3 Types of Planar Chromatography

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a container with a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture, which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.



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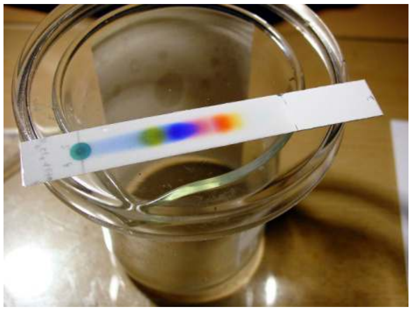


(ii)

Figure 1.2 (i) Paper Chromatography (ii) Thin Layer Chromatography.

Thin layer chromatography (TLC) is a widely employed laboratory technique used to separate different biochemicals on the basis of their size and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. TLC is very versatile; multiple samples can be separated simultaneously on the same layer, making it very useful for screening applications such as testing drug levels and water purity. Possibility of cross-contamination is low since each separation is performed on a new layer. Compared to paper, it has the advantage of faster runs, better separations, better quantitative analysis, and the choice between different adsorbents. For even better resolution and faster separation that utilizes less solvent, high-performance TLC can be used. An older popular use had been to differentiate chromosomes by observing distance in gel (separation of was a separate step).

2. THIN LAYER CHROMATOGRAPHY (THC)

2.1 Plate Preparation

TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulfate (gypsum) and water. This mixture is spread as thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and activated by heating in an oven for thirty minutes at 110 °C. The thickness of the absorbent layer is typically around 0.1 – 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC.

2.2 Generalized Concept

After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. The mobile phase has different properties from the stationary phase. For example, with silica gel, a very polar substance, non-polar mobile phases such as heptane are used. The mobile phase may be a mixture, allowing chemists to fine-tune the bulk properties of the mobile phase.

After the experiment, the spots are visualized. Often this can be done simply by projecting ultraviolet light onto the sheet; the sheets are treated with a phosphor, and dark spots appear on the sheet where compounds absorb the light impinging on a certain area. Chemical processes can also be used to visualize spots; anisaldehyde, for example, forms colored adducts with many compounds, and sulfuric acid will char most organic compounds, leaving a dark spot on the sheet.

2.3 Technique and Mechanism

The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products. Plates can be labeled before or after the chromatography process using a pencil or other implement that will not interfere or react with the process.

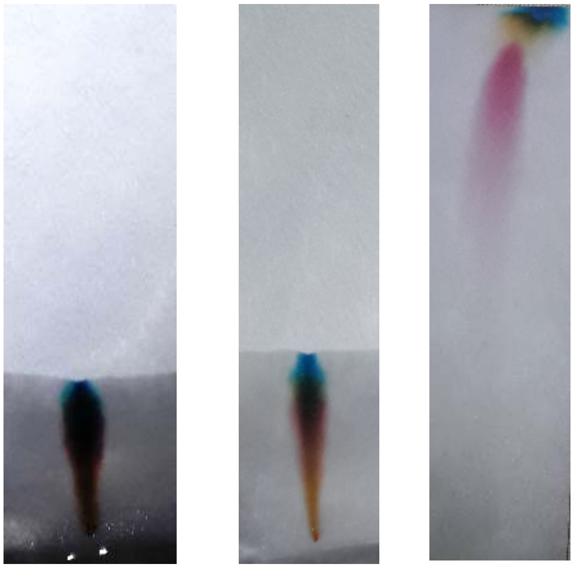
To run a thin layer chromatography plate, the following procedure is carried out:

Figure 2.1

Chromatography Paper dipped in Ethanol Solution: Initiation of Soaking-wetting and gradual upward upliftment of solution in the paper due to capillary action.

1) Using a capillary, a small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The solvent is allowed to completely evaporate off to prevent it from interfering with sample's interactions with the mobile phase in the next step. If a non-volatile solvent was used to apply

the sample, the plate needs to be dried in a vacuum chamber. This step is often repeated to ensure there is enough analyte at the starting spot on the plate to obtain a visible result. Different samples can be placed in a row of spots the same distance from the bottom edge, each of which will move in its own adjacent lane from its own starting point.



(i) *(ii)* *(iii)*

Figure 2.2 (i) (ii)

Observable Colour Seperation and Completion of Chromatography

2) A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. A strip of filter paper (aka "wick") is put into the chamber so that its bottom touches the solvent and the paper lies on the chamber wall and reaches almost to the top of the container. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend the filter paper and saturate the air in the chamber. (Failure to saturate the chamber will result in poor separation and non-reproducible results).

3) The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and

the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate (elutes the sample). The plate should be removed from the chamber before the solvent front reaches the top of the stationary phase (continuation of the elution will give a misleading result) and dried.

4) Without delay, the solvent front, the furthest extent of solvent up the plate, is marked.

5) The plate is visualized. As some plates are pre-coated with a phosphor such as zinc sulfide, allowing many compounds to be visualized by using ultraviolet light; dark spots appear where the compounds block the UV light from striking the plate. Alternatively, plates can be sprayed or immersed in chemicals after elution. Various visualising agents react with the spots to produce visible results.

Separation Process and Principle

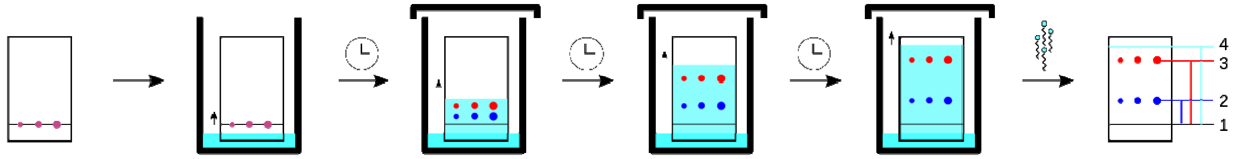


Figure 2.3 *Development of a TLC plate, a purple spot separates into a red and blue spot*

Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase and because of differences in solubility in the solvent. By changing the solvent, or perhaps using a mixture, the separation of components (measured by the Rf value) can be adjusted. Also, the separation achieved with a TLC plate can be used to estimate the separation of a flash chromatography column. (A compound elutes from a column when the amount of solvent collected is equal to 1/Rf.) Chemists often use TLC to develop a protocol for separation by chromatography and use TLC to determine which fractions contain the desired compounds.

Separation of compounds is based on the competition of the solute and the mobile phase for binding sites on the stationary phase. For instance, if normal-phase silica gel is used as the stationary phase, it can be considered polar. Given two compounds that differ in polarity, the more polar compound has a stronger interaction with the silica and is, therefore, better able to displace the mobile phase from the available binding sites. As a consequence, the less polar compound moves higher up the plate (resulting in a higher Rf value). If the mobile phase is changed to a more polar solvent or mixture of solvents, it becomes better at binding to the polar plate and therefore displacing solutes from it, so all compounds on the TLC plate will move higher up the plate. It is commonly said that "strong" solvents (eluents) push the analyzed compounds up the plate, whereas "weak" eluents barely move them. The order of strength/weakness depends on the coating (stationary phase) of the TLC plate. For silica gel-coated TLC plates, the eluent strength increases in the following order: perfluoroalkane (weakest), hexane, pentane, carbon tetrachloride, benzene/toluene, dichloromethane, diethyl ether, ethyl acetate, acetonitrile, acetone, 2-propanol/n-butanol, water, methanol, triethylamine, acetic acid, formic acid (strongest). For C18-coated plates the order is reverse. In other words, when the stationary phase is polar and the mobile phase is non-polar, the method is normal-phase as opposed to reverse-phase. This means that if a mixture of ethyl acetate and hexane as the mobile phase is used, adding more ethyl acetate results in higher Rf values for all compounds on the TLC plate. Changing the polarity of the mobile phase will normally not result in reversed order of running of the compounds on the TLC plate. An eluotropic series can be used as a guide in selecting a mobile phase. If a reversed order of running of the compounds is desired, an apolar stationary phase should be used, such as C18-functionalized silica.

3. THC AND ITS APPLICATIONS

3.1 Analysis

As the chemicals being separated may be colorless, several methods exist to visualize the spots:

1) Fluorescent analytes like quinine may be detected under blacklight (366 nm)
2) Often a small amount of a fluorescent compound, usually manganese-activated zinc silicate, is added to the adsorbent that allows the visualization of spots under UV-C light (254 nm). The adsorbent layer will thus fluoresce light-green by itself, but spots of analyte quench this fluorescence.
3) Iodine vapors are a general unspecific color reagent
4) Specific color reagents into which the TLC plate is dipped or which are sprayed onto the plate exist.
-Potassium permanganate - oxidation
-Bromine
5) In the case of lipids, the chromatogram may be transferred to a PVDF membrane and then subjected to further analysis, for example mass spectrometry, a technique known as Far-Eastern blotting.

Once visible, the Rf value, or retardation factor, of each spot can be determined by dividing the distance the product traveled by the distance the solvent front traveled using the initial spotting site as reference. These values depend on the solvent used and the type of TLC plate and are not physical constants.

3.2 Mathematical Quantification

To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase. (The mobile phase must not be allowed to reach the end of the stationary phase.) This ratio is called the retardation factor (Rf). In general, a substance whose structure resembles the stationary phase will have low Rf, while one that has a similar structure to the mobile phase will have high retardation factor. Retardation factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase. For this reason, chemists usually apply a sample of a known compound to the sheet before running the experiment.

$$\textit{Rf Value} \quad = \frac{\textit{distance from baseline travelled by solute}}{\textit{distance from baseline travelled by solvent (solvent front)}}$$

3.3 Applications

Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye composition of fibers in forensics, assaying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents

A number of enhancements can be made to the original method to automate the different steps, to increase the resolution achieved with TLC and to allow more accurate quantitative analysis. This method is referred to as HPTLC, or "high-performance TLC". HPTLC typically uses thinner layers of stationary phase and smaller sample volumes, thus reducing the loss of resolution due to diffusion.

3.3.1 Characterization

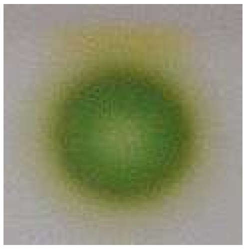
In organic chemistry, reactions are qualitatively monitored with TLC. Spots sampled with a capillary tube are placed on the plate: a spot of starting material, a spot from the reaction mixture, and a cross-spot with both. A small (3 by 7 cm) TLC plate takes a couple of minutes to run. The analysis is qualitative, and it will show if the starting material has disappeared, i.e. the reaction is complete, if any product has appeared, and how many products are generated (although this might be underestimated due to co-elution). Unfortunately, TLCs from low-temperature reactions may give misleading results, because the sample is warmed to room temperature in the capillary, which can alter the reaction—the warmed sample analyzed by TLC is not the same as what is in the low-temperature flask. One such reaction is the DIBALH reduction of ester to aldehyde.

In one study TLC has been applied in the screening of organic reactions, for example in the fine-tuning of BINAP synthesis from 2-naphthol. In this method, the alcohol and catalyst solution (for instance iron (III) chloride) are placed separately on the base line, then reacted, and then instantly analyzed.

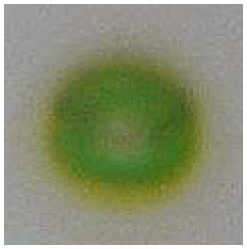
A special application of TLC is in the characterization of radiolabeled compounds, where it is used to determine radiochemical purity. The TLC sheet is visualized using a sheet of photographic film or an instrument capable of measuring radioactivity. It may be visualized using other means as well. This method is much more sensitive than the others and can be used to detect an extremely small amount of a compound, provided that it carries a radioactive atom.

3.3.2 Isolation

Since different compounds will travel a different distance in the stationary phase, chromatography can be used to isolate components of a mixture for further analysis. The separated compounds each occupying a specific area on the plate, they can be scraped off (along with the stationary phase particles) and dissolved into an appropriate solvent. As an example, in the chromatography of an extract of green plant material (for example spinach) shown in 7 stages of development, Carotene elutes quickly and is only visible until step 2. Chlorophyll A and B are halfway in the final step and lutein the first compound staining yellow. Once the chromatography is over, the carotene can be removed from the plate, extracted into a solvent and placed into a spectrophotometer to determine its spectrum. The quantities extracted are small and a technique such as column chromatography is preferred to separate larger amounts.


Step 1

Step 2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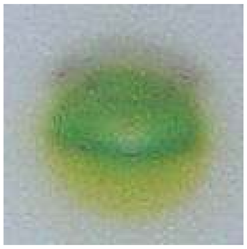
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Step 5

Step 6

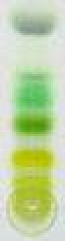
Step 7

Figure 3.1 *Chromatography of Chlorophyll (Source: Wikipedia, Florian Siebeck (Flo~commonswiki) assumed based on copyright claims)*

3.3.3 Examining Reactions

TLC is also used for the identification of the completion of any chemical reaction. To determine this it is observed that at the beginning of a reaction the entire spot is occupied by the starting chemicals or materials on the plate. As the reaction starts taking place the spot formed by the initial chemicals starts reducing and eventually replaces the whole spot of starting chemicals with a new product present on the plate. The formation of an entirely new spot determines the completion of a reaction.

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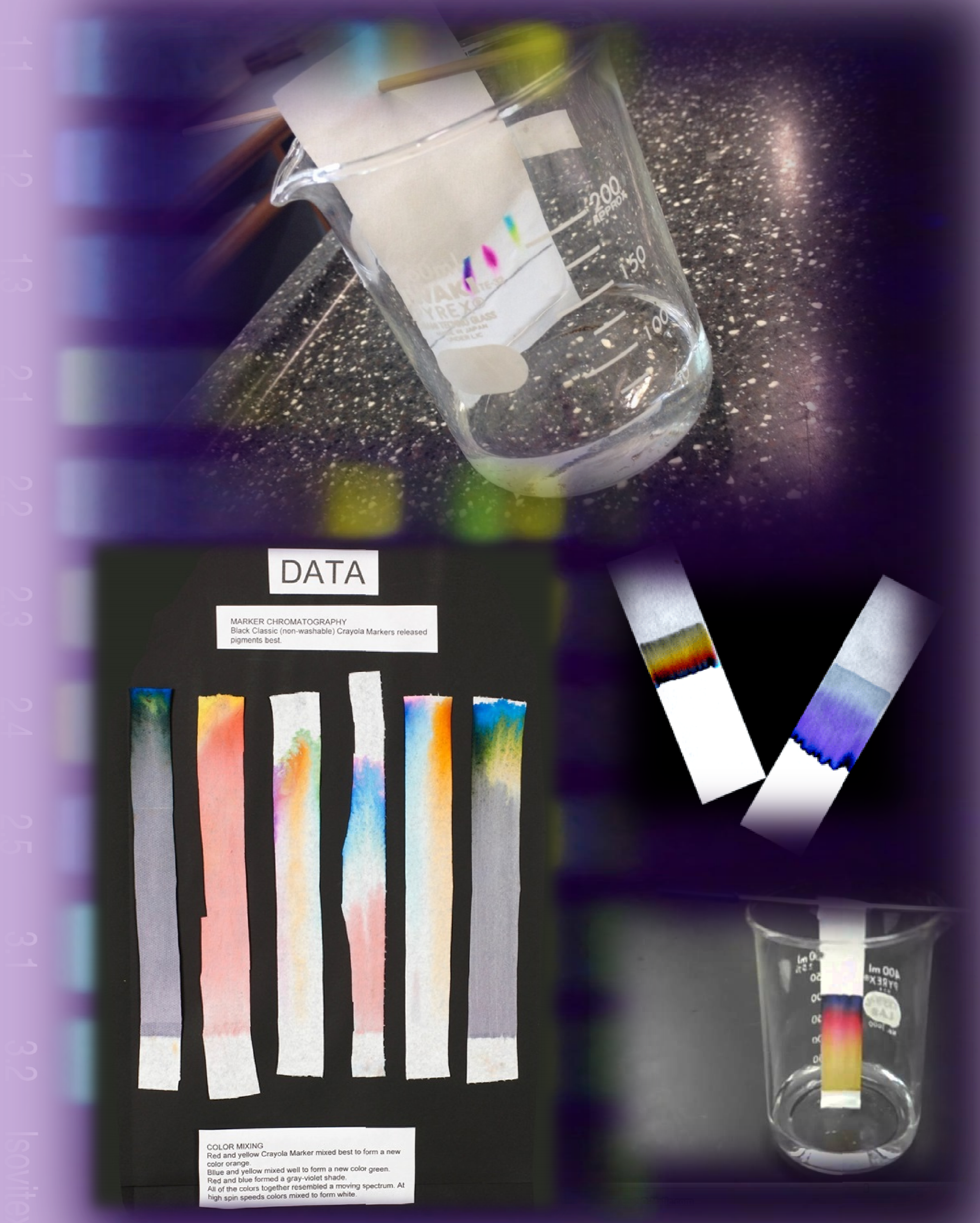

DATA

MARKER CHROMATOGRAPHY
Black Classic (non-washable) Crayola Markers released pigments best.

COLOR MIXING
Red and yellow Crayola Marker mixed best to form a new color orange.
Blue and yellow mixed well to form a new color green.
Red and blue formed a gray-violet shade.
All of the colors together resembled a moving spectrum. At high spin speeds colors mixed to form white.

PROJECT FOR SUBMISSION AT KENDRIYA VIDYALAYA MANKHURD FOR ALL INDIA SENIOR SCHOOL CERTIFICATE EXAMINATION CONDUCTED BY THE CENTRAL BOARD OF SECONDARY EDUCATION FOR THE YEAR 2018-2019